Atty. Docket No.: 046264-065331 PATENT
(Formerly 219781/2042)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Slepnev	Examiner:	Bertagna, A.M.
Serial No.:	10/600,201		
Filed:	June 20, 2003	Group Art Unit:	1637
Entitled:	Methods of Detecting Sequence Differences		
		Conf. No.:	5135

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF DR. VLADIMIR SLEPNEV UNDER 37 C.F.R. 1.132

I declare:

1. I, Vladimir I. Slepnev, hold the position of Chief Scientific Officer at Primera BioSystems, Inc., Mansfield, MA.

2. I hold a Master's Degree in Chemistry from Moscow State University and a Ph.D. in Biochemistry from the Russian Research Center of Molecular Diagnostics and Therapy (Moscow, Russia). I have performed post-doctoral research at Institute Pasteur (France) and at Yale University (New Haven, CT), where I also worked as a junior faculty member. I am an author on 32 peer-reviewed literature publications. A copy of my Curriculum Vitae is attached.

3. I am an inventor on the above-noted U.S. patent application.

4. I have read the Office Action issued July 9, 2007 in the above-noted patent application, and I understand that the Examiner has rejected claims 1-73 as either lacking novelty over the teachings of Myakishev et al. (2001, Genome Res. 11: 163-169) or obvious over various combinations of Myakishev et al. in view of Piggee et al. (1997, J. Chromatog. A. 781: 367-375), Wiesner et al. (1992, Biochem. Biophys. Res. Commun. 183: 553-559) and Nolan et al. (U.S. 6,287,766).

With respect to the primary reference, Myakishev et al., the Examiner stated:

Regarding claim 1, Myakishev teaches a method of determining a given nucleic acid sample, the identity of the nucleotide at a known polymorphic site (see Figure 2 and Results section, pages 163-165), said method comprising:

a) subjecting to an amplification regimen a population of primer extension products generated from a nucleic acid sample (Figure 2, top panel, where extension with the tailed allele-specific primers occurs in round 1 of the amplification to generate the population of primer extension products; see also page 164, col. 2; steps 3-4 shown in Figure 2 and page 164, col. 2 teach amplification of these primer extension products),

each primer extension product comprising a tag sequence, which tag sequence specifically corresponds to the presence of one specific nucleotide at a known polymorphic site (page 164, column 1, teaches that the allele-specific primer contains a different 21-base tail at the 5'end),

wherein said amplification regimen is performed using an upstream amplification primer (the "reverse primer" of page 163, col. 2 and page 164, col. 2) and a set of distinguishably labeled downstream amplification primers (the two energy-transfer (ET) primers of Fig. 2, see also page 164, col. 2), each member of said set of downstream amplification primers comprising a said tag sequence comprised by a member of said population of primer extension products and a distinguishable label (see Fig. 1 for the structure of the ET primers), wherein each distinguishable label specifically corresponds to the presence of a specific nucleotide at said polymorphic site (Fig. 2 teaches red and green labels for different mutations)

b) detecting incorporation of a distinguishable label into a nucleic acid molecule, thereby to determine the identity of the nucleotide at said polymorphic site (see Fig. 2 and pages 164-165).

With regard to independent claim 18, the Examiner stated:

Regarding claim 18, Myakishev teaches a method of determining, for a given nucleic acid sample, the identities of the nucleotides at a set of known polymorphic sites to be interrogated (see Fig. 2 and pages 163-165; note that 9 SNPs were tested), said method comprising:

a) subjecting to an amplification regimen, a population of primer extension products generated from a nucleic acid sample (Figure 2, top panel, where extension with the tailed primers occurs in round 1 of the amplification to generate the population of primer extension products; also page 164, col. 2; steps 3-4 shown in Figure 2 and page 164, col. 2 teach amplification of these primer extension products),

each primer extension product comprising a member of a set of tag sequences, which tag sequence specifically corresponds to the presence of one

specific nucleotide at a known polymorphic site (page 164, col. 1 teaches that the allele-specific primers contain different 5'tags),

wherein said amplification regimen is performed using one upstream amplification primer for each sequence comprising a known polymorphic site to be interrogated (the reverse primer, page 163), and a set of distinguishably labeled downstream amplification primers (the ET primers; Fig. 2),

each member of said downstream amplification primers comprising a said tag sequence comprised by a member of said population of primer extension products (see Figure 1) and a distinguishable label that specifically corresponds to the presence of a specific nucleotide at said polymorphic site (see Figures 1 & 2), and where said upstream amplification primers are selected such that each polymorphic site of said set of known polymorphic sites to be interrogated corresponds to a distinctly sized amplification product (see page 164, col. 1, where the upstream primer was hybridized at different points on the template to produce differently sized amplicons)

b) detecting incorporation of a distinguishable label in distinctly sized amplification products, thereby to determine the identity of the nucleotide at each said polymorphic site (see Fig. 2 and also the Methods section, page 168, where fluorescence is detected).

With regard to independent claim 34, the Office Action states:

Regarding claim 34, Myakishev teaches a method of determining, for a given nucleic acid sample, the identities of the nucleotides at a set of known polymorphic sites to be interrogated (see Fig. 2 and pages 163-165; note that 9 SNPs were tested), said method comprising:

a) subjecting to an amplification regimen, a population of primer extension products generated from a nucleic acid sample (Figure 2 and page 164, col. 2), each primer extension product comprising a first tag sequence (generated by extension of the reverse primer) or its complement and a member of a set of second tag sequences or its complement (the 21 nt tail added in step 1 of the reaction), the presence of which second tag sequence or its complement specifically corresponds to the presence of one specific nucleotide at a known polymorphic site (page 164, col. 1), wherein for each polymorphic site in said set of polymorphic sites, said first tag sequence is located at a distinct distance of 5' of said polymorphic site, relative to the distance of said first tag sequence from a polymorphic site on molecules in said sample containing other polymorphic sites (page 164, col. 1 teaches that the reverse primer may hybridize at different positions on the template), wherein said amplification regimen is performed using an upstream amplification primer comprising said first tag sequence (the reverse primer, page 163), and a set of distinguishably labeled downstream amplification primers (the ET primers), each member of said set of downstream amplification primers comprising a said tag sequence comprised by a member of said

population of primer extension products (the tail sequence; see Fig. 1) and a distinguishable label that specifically corresponds to the presence of a specific nucleotide at said polymorphic site (see Fig. 1 and 2), and wherein said upstream amplification primers are selected such that each polymorphic site of said set of known polymorphic sites to be interrogated corresponds to a distinctly sized amplification product (page 164, col. 1 teaches hybridization of the reverse primer at different locations on the template)

b) detecting incorporation of a distinguishable label in distinctly sized amplification products, thereby to determine the identity of the nucleotide at each said polymorphic site (page 168, col. 1).

5. I have read the Myakishev et al., Piggee et al., Wiesner et al., and Nolan et al. references. Based on the following analysis of the teachings of the cited references, and particularly the teachings of the Myakishev et al. and Wiesner et al. references, I cannot agree with the conclusion that the claimed invention lacks novelty over or is obvious over the proposed combinations of Myakishev et al., Piggee et al., Wiesner et al., and Nolan et al.

6. The method presented and claimed in the pending patent application allows simultaneous analysis of multiple SNPs in the same amplification reaction. The identity of the SNP sites is encoded by the size of PCR products and the identity of the nucleotide in the SNP site is encoded by fluorescent color. This method is particularly useful for analysis of a small set of SNPs (10-20 SNPs) in a single reaction. In addition, the method can also provide quantitative information, which could be used to quantify SNPs of interest, for example for testing of drug resistant alleles, oncogenic markers, etc.

The method of Myakishev uses fluorescent color to distinguish between two alleles within the same SNP site to establish the identity of the nucleotide in particular SNP site. However, the method does not teach multiplexing in terms of simultaneous analysis of a set of SNPs in the same reaction. Following the logic of their assay, testing additional SNP sites in the same assay would require use of additional fluorescent markers, so that one distinguishable marker is used for each nucleotide possible at the SNP site, and therefore any additional SNP will require 2 additional fluorescent markers. There is a possibility that such approach may work for 2 SNPs in a one assay (wherein 4 fluorescent markers are used), but there are problems even at this minimum level of multiplexing, and the approach is unlikely to succeed for more than one SNP in one assay, for a number of reasons. First, fluorescence takes place in a rather limited

range of the optical spectrum resulting in significant overlap in excitation and emission spectra of fluorescent markers. In practice, it is impossible to find 4 fluorescent dyes without significant overlap in the emission spectrum. While it is possible to discriminate 4-5 dyes in ideal conditions, distinguishing a greater number of fluorescent markers becomes insolvable, particularly when concentrations of individual markers are unknown (as occurs in PCR). Multiplexing with color using the method of Myakishev et al. with more than two fluorescent markers requires deconvolution of a complex fluorescent spectrum, created by overlapping emission of multiple fluorescent markers which are often present at unknown and unequal concentration (as a result of potentially uneven efficiency of PCR amplification). This is the main reason limiting the multiplexing capacity of real-time PCR to 4 colors, and for the same reason, the method of Myakishev et al. can not be used for testing of more than 2 SNPs in the same assay. Since the authors did not teach multiplex analysis even for 2 SNPs in the assay, it is not clear and remains to be seen if this approach will actually work in any multiplex setting.

The most uncertainty comes from Myakishev et al.'s assay design, which uses un-quenching of quenched fluorescent marker in the course of PCR. In this design, the assay contains two forms of the same fluorescent marker with different emission intensity (one corresponding to the primer, and another to the product). Given that emission spectra of fluorescent markers are partially overlapping and that the ratio of quenched to un-quenched forms of particular marker is unknown (it needs to be calculated from the fluorescent emission intensity after subtracting the emission intensity coming from overlapping markers at the selected wavelength), it is not in any way clear that detecting genotypes of even two SNPs in the same reaction is feasible using the unquenching approach taught by Myakishev et al. This would require a special computational algorithm to separate measured fluorescent emission at multiple wavelengths into 4 fluorescent colors. Myakishev et al. did not teach such an algorithm. This problem does not exist for the two-color detection scheme used by Myakishev et al., where the dyes have significant spectral separation and signal intensity came be measured at just two wavelengths as described in the reference.

7. Given that the method of Myakishev et al is not amenable to multiplexing and was not intended to be, I have real difficulty to see how it could be combined with the reference of Piggee et al. to become capable of multiplexing. The multiplexing aspect suggested by Piggee et

al. describes multiplexed detection by CE of primer extension reactions using fluorescently-labeled terminating nucleotides and primers of different lengths, wherein the length of the primer encodes the identity of the SNP. The claimed invention involves multiplexed detection of the products of multiplex PCR amplification separated by size or/and by charge, wherein the products of PCR amplification are of different length, encoding the identity of the SNP. The Piggee et al. reference does not teach multiplex amplification. Instead it describes individual PCR amplification for each SNP, purification of amplified PCR product (which is a key step in the procedure), and putting together several PCR products for multiplex primer extension. The Piggee et al. reference teaches the use of primers of different length as opposed to using distinguishably sized PCR products in the claimed invention. Since the type of the molecule that is being separated is inherently linked to the labeling chemistry, the difference is significant. The process described by claimed invention relies on signal generation during multiplex PCR, and success of multiplex PCR improves with the use of the primers with similar melting temperatures, which usually are primers with similar length and composition. Multiplexing using amplicon length allows a greater degree of multiplexing (sizes up to 1000b can be used for separation), allows more flexibility of assay design and uniformity of PCR amplification procedure. For practical purposes a size differential of at least 5 bases between two different nucleic acids is preferred to accommodate for mobility shift (introduced by fluorescent marker) and to provide for enough resolution between highly abundant and rare nucleic acids.

In addition, the processes described by Piggee and Myakishev are fundamentally different in terms of signal-generating chemistry: Piggee et al. relies on the addition of non-extendable bases to the 3'-end of the specific primer sequence, rendering the extension product non-amplifiable; and the Myakishev et al. method incorporates a specific sequence element at the 5'-end of the amplicon and uses the sequence for signal-generating PCR amplification. Given these fundamental differences in signal generating chemistry, I can not envision a chemical process that would successfully combine both methods.

8. In my opinion, one cannot use DNA quantification described by Wiesner in combination with capillary electrophoresis for a number of reasons. First, the method described by Wiesner relies upon the removing of a known volume of reaction sample containing radioactive nucleotides at subsequent cycles of PCR, separating the entire aliquot by electrophoresis,

measuring radioactivity incorporated in the PCR products, plotting the calculated copy number at each cycle and then extrapolating the resulting function to the cycle zero to obtain the initial number of nucleic acid target molecules in the sample. The method described by Wiesner relies on two key conditions: A) knowledge of the exact volume of the aliquot subjected to separation; and B) the ability to measure precisely the absolute quantity of the PCR product band at the given cycle. The absolute determination of target template taught by Wiesner will not work when applied to capillary electrophoresis and, particularly with fluorescent detection, because these key conditions will not be satisfied as required for the linear regression calculations that are central to the Wiesner method.

A) Knowledge of the exact volume of aliquot subjected to separation is required by Wiesner.

Wiesner teaches in its "Experimental Procedure" section on page 554:

> "Aliquots of 1 µl were taken from the reaction after each consecutive cycle and loaded on 2% agarose gels stained with ethidium bromide (9). ....

In its "Calculations" section on page 554, the Wiesner et al. reference states:

> "The concentration of COX I product (moles/µl) produced in consecutive cycles was calculated from the incorporated radioactivity (cpm/µl), the specific activity of the precursor dCTP (cmp/mol), determined in 1 µl aliquots of the reaction mixture and the number of cytosine moieties in the product (144 cytosins) according to
>
> $$\text{moles}/\mu\text{l} = \frac{\text{cpm}/\mu\text{l}}{\text{cpm/mol} \times 144} \quad \text{(eq. 1)}$$
>
> "

Thus, the volume of sample (here, 1 µl) is a critical variable in the method. Further, Wiesner et al. expresses the concentration of amplified product in terms of moles produced/µl – see, e.g., Fig. 1, page 555 and Fig. 2, page 556.

It is clear from the description that the method is based on the exact knowledge of the volume of the aliquot subjected to agarose gel electrophoretic separation, which becomes the basis for all calculations. Capillary electrophoresis (CE) differs from slab gel electrophoresis in the way that the sample is loaded into the separation medium. Whereas the entire volume of the

aliquot placed into the slab gel goes into the separation, only a small and unknown portion of the aliquot placed for injection into the capillary actually enters the capillary.

In capillary electrophoresis there two ways to inject sample into a capillary: hydrodynamic injection and electrokinetic injection. Hydrodynamic injection, where sample is drawn into the capillary by creating a pressure differential between inlet and outlet ends of the capillary, is rarely used for nucleic acid separation due to the high viscosity of sieving polymer gels. The viscosity makes it very difficult to control the volume of the injected sample. It is nearly impossible to implement this mode of injection for multicapillary systems which are the preferred approach for nucleic acid CE.

In the electrokinetic mode of injection, a capillary is dipped into the sample aliquot together with an electrode, and by applying an electric field to the sample, negatively charged nucleic acids enter the capillary and migrate toward positively charged electrode placed at the outlet of the capillary. The amount of the nucleic acids in the sample actually injected into the capillary depends on the duration of the injection, potential of the applied electric field, ionic composition of the sample (components of PCR mixture: salts, nucleotides and primers compete for injection with nucleic acids),"stacking" effect at the border of separating polymer and sample solution, and electroosmotic force moving the liquid enclosed in the capillary in the opposite direction in the electric field. The upper limit on the amount of injected material is imposed by the requirement to maintain reasonable resolution of the separation (the "length" of injected solution plug should be a small fraction of the capillary length). Technically, it is impossible to inject a sample with a volume equivalent of more than 100 nl. More importantly, the exact "volume" of the injected sample using electrokinetic injection is unknown. To adapt the method of Wiesner to the use of CE in place of slab gel electrophoresis, one would have to measure the volume of the sample injected during CE, however the Wiesner reference does not provide a teaching for how to do that, and neither does the Piggee et al. reference, or any of the other references. As discussed above, such a measurement may well not be possible.

B) Wiesner requires precise measurement of the absolute amount of amplified PCR product contained within a separated peak.

In addition to requiring knowledge of the exact volume of sample electrophoresed, the method of Wiesner requires precise measurement of the absolute amount of amplified PCR product contained within a separated peak. In the method described by Wiesner, radioactive label is used to measure incorporation in the PCR product of interest. It is conceivable to consider that a direct adaptation of Wiesner's method to capillary electrophoresis could include an approach which would measure the amount of radioactive label in the peaks separated by CE. This could be accomplished by using an in-line radioactive detector or by collecting fractions during CE and then measuring radioactive label in the collected fractions corresponding to the specific PCR product. However, to my knowledge, no in-line radioactive detectors exist yet for CE separation of nucleic acids, and so far fraction collection for CE is used for qualitative rather than quantitative assessments of nucleic acids.

Another option for absolute quantification of nucleic acid in the separated peaks is UV photometry. Unfortunately, the small optical path of the capillary (50-100 microns ID) prohibits the use of this detection method for applications requiring highly sensitive detection, such as monitoring of PCR amplification.

The preferred detection method in CE separation of nucleic acids is fluorescent detection. This method, by definition, does not provide absolute quantification, and therefore it uses additional standards of known abundance for relative quantitative measurement of target analytes. In order to adapt the quantitation method of Wiesner to use capillary electrophoresis in place of slab gel electrophoresis, one would need to provide a reference standard or multiple standards comprising the same type of fluorophore with identical excitation and emission properties to the target PCR amplicon. Since nucleotide sequence context is well known to affect properties of fluorescent labels, special reference standards would have to be developed for each individual amplicon present in the same multiplex reaction. Wiesner, or Myakishev et al., Piggee et al. or Nolan et al., for that matter, do not teach design and validation of such standards. Moreover, I am not aware of any published reference that would teach practical implementation of such standards for absolute quantification of nucleic acids by capillary electrophoresis.

It is my strong opinion that quantitation as taught by Wiesner cannot be achieved using capillary electrophoresis. It is my strong opinion that the method of Wiesner requires specific

means described in the reference and that capillary electrophoresis cannot be simply substituted for slab gel electrophoresis if one expects to use the template nucleic acid quantitation approach that is central to the teachings of the Wiesner reference.

In view of the above, the proposed combination of teachings of Wiesner, Myakishev et al. and Piggee et al. that are central to the claim rejections will not provide function necessary to meet the requirements of the claimed invention.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

01/09/2008

Date

Vladimir I. Slepnev